Stimulation of Erythropoiesis by In Vivo Gene Therapy: Physiologic Consequences of Transfer of the Human Erythropoietin Gene to Experimental Animals Using an Adenovirus Vector

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Erythropoietin (Epo), a 30.4-kD glycoprotein, is the principal regulator of erythropoiesis. To evaluate the concept that in vivo gene transfer might be used as an alternative to recombinant human Epo (rhEpo) in applications requiring a 1- to 3-week stimulation of erythropoiesis, the replication-deficient recombinant adenovirus AdMLP.Epo was constructed by deleting the majority of E1 from adenovirus type 5, and replacing E1 with an expression cassette containing the adenovirus type 5 major late promoter (MLP) and the human Epo gene, including the 3' cis-acting hypoxia response element. In vitro studies showed that infection of the human hepatocyte cell line Hep3B with AdMLP.Epo resulted in a 15-fold increase in Epo production in 24 hours that was enhanced to 116-fold in the presence of a hypoxic stimulus. One-time in vivo administration of AdMLP.Epo (7 × 10^9 plaque-forming units/kg) to the peritoneum of cotton rats caused a marked increase in red blood cell production, with a 2.6-fold increase in bone marrow erythroid precursors by day 4, and sevenfold increase in reticulocyte count by day 7. The hematocrit increased gradually, with a maximum of 64% ± 4% at day 14 (compared with an untreated baseline of 46% ± 2%), and a level of 55% ± 1% at day 24. Furthermore, one-time subcutaneous administration of AdMLP.Epo caused an increase in hematocrit that peaked at 14 days (57% ± 2%) and was still elevated at day 42. Hematocrit level in animals receiving subcutaneous administration of AdMLP.Epo sustained a long-term increase compared with animals receiving intraperitoneal administration. In the context of these observations, gene therapy with single administration of an adenovirus vector containing the human EPO gene may provide a means of significantly augmenting the circulating red blood cell mass over the 1- to 3-week period necessary for many clinical applications. © 1994 by The American Society of Hematology.

MATERIALS AND METHODS

Construction of AdMLP.Epo. An E1 replication-deficient recombinant adenovirus (Ad) containing the human Epo gene (AdMLP.Epo) was constructed using homologous recombination to combine an expression cassette containing the adenovirus type 5 (Ad5) major late promoter (MLP) and the Epo gene with the pJM17 plasmid containing the Ad5 genome (Fig 1). The final AdMLP.Epo construct includes the Ad5 MLP driving the human Epo gene, containing 217 bp 5' to the translation start, all five coding exons and four introns, and 831 bp 3' to the translation stop. The region 3' to the stop codon includes a 150-bp segment downstream from the polyadenylation site that functions as a cis-acting hypoxia responsive enhancer element, permitting evaluation of the regulation of expression of the construct in cultured human hepatoma cell line Hep3B (ATCC HB8064).

Plaques of AdMLP.Epo were isolated and identified by polymerase chain reaction (PCR) after 14 days, and a single plaque was amplified by propagation in 293 cells. The 293 cells are a modified line of human embryonic kidney cells.
Recombinant virus was released from infected 293 cells by five cycles of freeze-thawing. AdMLP.Epo was further purified by CsCl gradient centrifugation and dialysis against virus suspension buffer (10 mmol/L Tris-Cl, pH 7.4, 1 mmol/L MgCl2, 10% glycerol), and stored at -70°C. Titters of the viral stock were determined by plaque assay using 293 cells. All preparation of AdMLP.Epo were evaluated by enzyme linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN) to ensure there was no contaminating human Epo.

**Function of AdMLP.Epo in vitro.** To evaluate the ability of AdMLP.Epo to direct the production of human Epo in vitro, AdMLP.Epo (10⁶ plaque-forming units [pfu]) was added to 10⁶ COS-7 cells (ATCC CRL 1651) and the cells were cultured for 90 minutes, 37°C, in 1 mL of infection media (improved minimal essential medium containing 2 mmol/L glutamine, penicillin [50 U/mL], streptomycin [50 μg/mL], and 2% heat-inactivated fetal bovine serum [FBS]). As controls, parallel cultures were incubated with the replication-deficient recombinant adenovirus Ad-α1AT containing the human α1-antitrypsin cDNA or with virus suspension buffer alone. The medium was replaced with complete media (same as infection media, but with 10% FBS), and the incubation continued for 24 hours. The supernatants were evaluated for human Epo using an Epo-specific ELISA (detectable threshold 2 μU/mL).

To show that the AdMLP.Epo construct would respond to hypoxic conditions and enhance the expression of the Epo gene, AdMLP.Epo (10⁶ pfu), the control vector Ad-α1AT (10⁶ pfu), or the same volume of virus suspension buffer was added to 10⁶ Hep3B (ATCC HB8064) cells in 500 μL of infection media. After 90 minutes at 37°C, 5 μL of complete media was added. After 24 hours, the media was replaced with either complete media or complete media + 50 μmol/L of CsCl (to mimic hypoxic conditions). After another 24 hours the supernatants were evaluated for Epo by ELISA.

**Function of AdMLP.Epo in vivo.** To evaluate the ability of AdMLP.Epo to direct the synthesis of Epo in vivo, cotton rats (Sigmodon hispidus; 120 to 160 g body weight) were anesthetized with methoxyflurane inhalation (Metofane; Pitman-Moore, Mundelein, IL), and 7 × 10⁷ pfu/kg of AdMLP.Epo, Ad-α1AT (as a control vector), or an equivalent volume of virus suspension buffer diluted with phosphate-buffered saline (PBS) pH 7.4 to 500 μL/animal were directly instilled into the peritoneal cavity. The animals were killed after methoxyflurane anesthesia by exsanguination before (0 time) or at days 4, 7, 14, and 24 after administration of the virus. Samples of BM (from the femur) and blood (by cardiac puncture) were obtained. For each animal, two smears of BM stained with Wright-Giemsa and two smears of blood stained with new methylene blue N solution were prepared. BM and blood smears were evaluated by light microscopy analysis before (0 time), and 4, 7, 14, and 24 days later for the proportion of nucleated BM cells that were erythroid precursors (minimum of 500 cells counted in duplicate samples for each animal), the proportion of RBCs that were reticulocytes (minimum of 500 cells counted in duplicate samples for each animal), and the hematocrit (percent volume of blood occupied by RBCs; duplicate samples for each animal). A minimum of two animals were evaluated at each data point with duplicate samples from each animal.

For comparison of the hematocrit response to AdMLP.Epo, a single dose of recombinant human erythropoietin (rhEpo; Amgen Inc, Thousand Oaks, CA; 50 to 2,000 U/kg in 0.025% rat serum) was administered to the peritoneum in the same fashion as AdMLP.Epo. For each data point, a minimum of two determinations of three animals were evaluated. As a control, untreated animals received 0.025% rat serum. To evaluate in vivo subcutaneous Epo gene transfer, 7 × 10⁷ pfu/kg of AdMLP.Epo, Ad-α1AT (as a control vector), or an equivalent volume of virus suspension buffer diluted with PBS up to 500 μL

human embryonal kidney cells transformed by adenovirus type 5. These cells contain 11% (3,960 bp) of the left-hand adenovirus type 5 genome. These 293 cells provide Ela and Elb region in trans, thus propagating infectious replication-deficient, recombinant adenovirus. Recombinant virus was released from infected 293 cells by
per animal were directly injected into subcutaneous tissue of cotton rats. The hematocrit was assayed before (time 0) and at days 7, 14, 21, 28, 35, and 42 after administration.

Further using Epo-specific ELISA, we measured human EPO levels in serum samples from animals treated with AdMLP.Epo, Ad-α1AT, or virus suspension buffer, before (time 0) and after administration at days 2, 4, 7, 14, 24, and 35. A minimum of three animals were evaluated at each data point with duplicate samples from each animal. We also compared serum human Epo level in animals administered AdMLP.Epo and animals treated with rhEpo (1,000 U/kg) intraperitoneally. Serum human Epo levels were evaluated before and after administration at days 1 and 2.

RESULTS

Function of AdMLP.Epo in vitro. Addition of AdMLP.Epo to COS-7 cells at 10⁶ pfu/10⁶ cells demonstrated expression of the human Epo gene, with the AdMLP.Epo-infected COS-7 cells secreting 17 ± 2 U of human Epo/24 hours as assessed by an ELISA (all data are presented as mean ± standard error of the mean; all statistical comparisons are made using the two-tailed Student’s t-test). In contrast, the supernatants of uninfected COS-7 cells, or COS-7 infected with the control vector Ad-α1AT had not contain detectable human Epo.

To evaluate the ability of the 3' cis-acting hypoxia-sensitive element in AdMLP.Epo to respond to a hypoxic stimulus and stimulate the heterologous constitutive Ad5 major late promoter to enhance expression of the EPO gene, the human hepatoma cell line Hep3B was exposed to AdMLP.Epo (100 pfu/cell) and the cells were maintained in culture with media alone or with media containing Co²⁺. As has been previously noted, untreated Hep3B cells produced small amounts of Epo (Fig 2). The amounts of Epo secreted did not change when the Hep3B cells were infected with the control adenovirus vector Ad-α1AT. In contrast, the amount of Epo secreted increased 15-fold when the Hep3B cells were infected with AdMLP.Epo. The endogenous EPO gene in the Hep3B cells responded to a cobalt stimulus (P < .001), as did the Hep3B cells infected with Ad-α1AT (P < .05). In marked contrast, the AdMLP.Epo-infected cells exposed to Co²⁺ had a large increase in Epo production, 22-fold compared with AdMLP.Epo-infected cells without Co²⁺ (P < .01), and 116-fold compared with uninfected Hep3B cells exposed to Co²⁺ (P < .001).

Function of AdMLP.Epo in vivo. To evaluate the ability of AdMLP.Epo to transfer the EPO gene in vivo with consequent expression of human EPO and erythropoiesis, AdMLP.Epo was administered to the peritoneum of cotton rats. The peritoneum was chosen because it has a large surface area (2 m² in the human), it is permeable to macromolecules of the size of EPO, and there is evidence that administration of rhEPO into the normal peritoneal cavity results in efficient transfer of Epo into the systemic circulation with consequent increase in RBC levels. The cotton rat was used because it is the experimental animal closest to human in regards to relative susceptibility to human adenovirus infection. Based on our experience that experimental animals develop circulating anti-adenovirus antibodies in response to systemic administration of human adenovirus vectors and that anti-adenovirus antibodies may limit adenovirus infection, we focused on the one-time administration of an adenovirus vector with the potential of using Epo gene therapy for short-term (1 to 3 weeks) applications.

Intraperitoneal administration of AdMLP.Epo was well tolerated. After preliminary studies showed that a maximum response would be achieved with 10⁶ to 10⁷ pfu/animal, 7 × 10⁶ pfu/kg were used for all subsequent studies.

In control cotton rats, the BM erythroid precursors represented 19% ± 1% of all nucleated hematopoietic marrow cells (Figs 3A and 4A). The same was true for animals receiving intraperitoneal administration of the control Ad vector Ad-α1AT, with values of 18% to 21% over a 24-day period (all values, P > .1 compared with control untreated animals) (Figs 3B and 4A). Strikingly, AdMLP.Epo-treated animals had a significant increase in the proportion of erythroid precursors throughout the observation period (4 to 24 days; all values with AdMLP.Epo, P < .001 compared with untreated controls and Ad-α1AT–treated animals) (Figs 3C and 4A).

Consistent with the expansion in numbers of marrow erythroid precursors induced by AdMLP.Epo, there was a marked reticulocytopsisis observed in the cotton rats receiving AdMLP.Epo, compared with untreated animals and those treated with Ad-α1AT (Figs 3D and 4B). In control, untreated cotton rats, reticulocytes represented an average of 2% ± 1% of all circulating RBCs. The same was true in animals receiving the control vector Ad-α1AT observed over a period of 24 days. In marked contrast, cotton rats receiving
AdMLP.Epo had reticulocyte proportions of 15% ± 1% at day 7, thereafter decreasing, but remaining threefold increased at day 14, and approximately twofold increased at day 24 ($P < .001$, compared AdMLP.Epo with controls).

Consistent with the marked stimulation of BM erythroid precursors and circulating reticulocytosis, intraperitoneal administration of AdMLP.Epo was associated with a marked increase in the hematocrit (Fig 4C). In control cotton rats, and in animals receiving Ad-α1AT, the average circulating hematocrit was 45% to 47%. In contrast, in animals receiving
AdMLP.Epo, the hematocrit began increasing by 4 days. By 7 days, it had reached 57% ± 1% (P < .001, compared with untreated and Ad-αIAT-treated animals) with a peak level of 64% ± 4% at day 14 (P < .005 compared with both controls). By 24 days, consistent with the decrease in the relative proportion of marrow erythroid precursors and the decrease in the reticulocyte count, the hematocrit had declined, but was still 18% higher than the baseline, with a level of 55% ± 1% (P < .003 compared with untreated and Ad-αIAT-treated animals).

The pattern of increase in hematocrit induced by the one-time intraperitoneal administration of AdMLP.Epo was similar to that observed with a one-time administration of purified recombinant human erythropoietin, but the response to AdMLP.Epo was much greater and longer lasting (Fig 4D). One-time intraperitoneal administration of rhEpo caused a dose-response augmentation in hematocrit with peak responses at day 14. However, even at doses of 2,000 U/kg, the peak hematocrit value at day 14 was only 55% ± 1%, compared with 46% ± 1% for controls and 64% ± 4% with AdMLP.Epo at the same time point (P < .03, AdMLP.Epo compared with rhEpo). Further, whereas the hematocrit of animals receiving 2,000 U/kg of rhEpo had returned to baseline by 24 days, the hematocrit was still elevated at day 24 in the animals receiving AdMLP.Epo (P < .05).

One-time subcutaneous administration of AdMLP.Epo caused an increase in hematocrit that peaked at day 14 (57% ± 2%) and the hematocrit was still elevated at day 42 (51% ± 1%) in the animals receiving AdMLP.Epo. Hematocrit level in animals receiving subcutaneous administration of AdMLP.Epo also sustained a long-term increase compared with animals receiving intraperitoneal administration (Fig 4E).

With 7 × 10⁸ pfu/kg AdMLP.Epo administered to the peritoneum, analysis of the serum 2 days later showed human Epo levels of 100 ± 9 U/mL and Epo was detected up to 24 days (Fig 4F). In contrast, untreated animals or animals infected with Ad-αIAT as a control had no human Epo detectable in serum. In the animals receiving a single administration of rhEpo (1,000 U/mL), serum human EPO was 182 ± 13 U/mL at 1 day and undetectable at 2 days.

**DISCUSSION**

The observations in the present study show that a replication-deficient adenovirus vector can be used for in vivo tran-
fer of a gene coding for a specific hematopoietic growth factor, with consequent expression of the gene product, and physiologic function of the growth factor at a distant site to expand specific BM precursors. To evaluate this concept, we used the Epo gene, the gene coding for the hematopoietic growth factor specific for erythroid precursors.17-19 The results confirm the power of adenovirus vectors to transfer genes in vivo, with one-time administration of the recombinant vector containing the human Epo gene resulting in more extensive augmentation of the circulating RBC mass than that observed with one-time administration of high doses of rhEpo. Based on these observations, it is likely that adenovirus vectors could be used to successfully transfer and express genes coding for a variety of hematopoietic growth factors, including granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF, macrophage CSF, interleukin-3 (IL-3), and stem cell factor.43,44

There are theoretical advantages of adenovirus-mediated in vivo gene transfer for clinical applications involving genes for hematopoietic growth factors. First, it is likely that adenovirus-mediated transfer of genes such as the human Epo gene would likely be less costly than the complex process of expressing the gene in vitro in mammalian cells and purifying the protein.45 In contrast, for adenovirus-mediated gene transfer, production costs could be limited to that of the vector, because the in vivo expression of the gene obviates the necessity to purify the protein. Second, for clinical applications requiring 2 to 3 weeks of augmentation of RBC mass, transfer of the Epo gene with an adenovirus vector would require only a single administration to obtain a satisfactory clinical response, whereas the pharmacokinetics of rhEpo could demand multiple administrations to achieve the same result. Third, although in vitro systems have been developed to produce several hematopoietic growth factors for experimental and clinical use, the clinical efficacy of novel gene products coding for hematopoietic growth factors could be rapidly evaluated in vivo in experimental animals and eventually humans using adenovirus-mediated gene transfer without having to produce and purify the gene product in vitro. Finally, adenovirus vectors can transfer genes to a variety of cell types in vivo, permitting diverse strategies for in vivo gene transfer of hematopoietic growth factors. In the present study the peritoneal mesothelium cells were used as the target for in vivo gene transfer; furthermore, we showed that subcutaneous tissue was a more convenient target organ for adenovirus gene transfer, but it should be possible to use other sites such as muscle, liver, blood vessel, or lung,29-32,46-67 depending on the clinical circumstances.

The theoretical disadvantages of using adenovirus-mediated in vivo gene transfer of the hematopoietic growth factors relate to two factors: (1) safety of in vivo administration of these vectors; and (2) immune responses to the adenovirus. In regard to the safety aspects of using replication-deficient recombinant adenovirus vectors, a variety of in vitro and in vivo studies have been performed to demonstrate that the risk of using such vectors is very low.43 Further, the initial clinical evaluation of replication-deficient adenovirus vectors carrying the human cystic fibrosis transmembrane conductance regulator (CFTR) cDNA for individuals with cystic fibrosis has demonstrated it is feasible to transfer the CFTR cDNA to the airway epithelium at doses that are safe.43 In regard to possible humoral immune responses, the major theoretical concern relates to the possibility that humoral immune responses to the adenovirus capsid proteins may limit the efficacy of repeat administration of such vectors. Although adenovirus vectors do stimulate anti-adenovirus humoral immunity in vivo in cotton rats and nonhuman primates,46 these experimental animals are sufficiently different from humans that definitive evaluation of the "immunity-eficacy" question for chronic clinical applications will have to await human clinical trials for each specific application. Based on this knowledge, the present study was designed specifically to evaluate relatively short-term, single administration (eg, 2 to 3 weeks) use. The data support the concept that the AdMLP.Epo vector can evoke expansion of the RBC mass over a 4- to 24-day period.

Finally, it is of interest that the expression of the Epo gene in the AdMLP.Epo vector was enhanced in vitro in hepatoma cells after exposure of the cells to Co2+. The vector was designed to contain the human Epo gene 3' hypoxia responsive enhancer element.9 The in vitro observation that this 3' hypoxia response enhancer element will function in an E1 recombination adenovirus with a constitutive viral promoter suggests that it should be possible to design adenovirus vectors with appropriate regulatory signals for specific clinical applications, and expect that such regulatory signals may function appropriately to control specific genes.

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